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GAS6 Induces Axl-mediated Chemotaxis of Vascular Smooth Muscle Cells*

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Atherosclerosis and arterial restenosis are disease processes involving the accumulation of vascular smooth muscle cells following vascular injury. Key events leading to these processes are migration and proliferation of these cells. Here, we demonstrate that GAS6, encoded by the growth arrest-specific gene 6, induces a directed migration (chemotaxis) of both rat and human primary vascular smooth muscle cells while showing only marginal mitogenic potential in human vascular smooth muscle cells. GAS6 stimulation induces Axl autophosphorylation in human vascular smooth muscle cells, indicating that specific GAS6-Axl interactions may be associated with GAS6-directed chemotaxis. To test this hypothesis, vascular smooth muscle cells overexpressing Axl were generated by gene transfer and assessed for their ability to migrate along a GAS6 gradient. These Axl overexpressors exhibited 2–5-fold increased sensitivity to GAS6-induced chemotaxis. Furthermore, vascular smooth muscle cells expressing the kinase dead mutant of Axl or exposure to the soluble Axl extracellular domain showed attenuated GAS6-induced migration. Taken together, these results suggest that GAS6 is a novel chemoattractant that induces Axl-mediated migration of vascular smooth muscle cells. The separation of mitogenesis from migration provided by this study may enhance the molecular dissection of cell migration in vascular damage.

Atherosclerosis and arterial restenosis is a consequence of accumulation of connective tissue in conjunction with proliferation and directed migration of vascular smooth muscle cells (VSMC)[1] (1). To evaluate potential *in vivo* effects of proliferation and directed migration of VSMC following treatment of agonists or growth factors, primary, cultured VSMC have proven to be an excellent *in vitro* model system.

In cultured rat VSMC, GAS6, encoded by the growth arrest-specific gene 6 (*gas*6), was identified and characterized as an important growth-potentiating factor whose expression is up-regulated after serum starvation (2–4). GAS6 possesses a 44% sequence identity with protein S, an anti-coagulation factor (4). In quiescent VSMC, GAS6 stimulation specifically potentiates proliferation induced by Ca^{2+}-mobilizing receptors indicating that GAS6 may be involved in regulating signaling pathways mediated by heterotrimeric guanine nucleotide-binding (G) protein-coupled receptors. GAS6 alone, however, is able to prevent growth arrest-induced apoptosis in these cells (5). We have identified GAS6 to be the ligand for Axl, a member of a tyrosine kinase receptor family whose extracellular domains resemble cell adhesion molecules (6–13). It was subsequently demonstrated that GAS6 is also a ligand for Sky, an Axl-related receptor tyrosine kinase (14–16). In addition, it was recently shown that GAS6 may be the ligand for another Axl family member, Mer, although the affinity of the GAS6-Mer interaction is much lower than that for Axl and Sky (17). In rat VSMC, a high affinity, specific binding site for GAS6 was characterized and the molecular weight of the cross-linked complex coincided with that of the Axl·GAS6 complex (18). In support of the hypothesis that the GAS6/Axl pathway is involved in G protein receptor-mediated signaling events is recent evidence indicating that Axl expression is up-regulated by G protein-coupled receptor agonists both in cultured VSMC and *in vivo* following balloon injury.[2] Thus, these results suggest that GAS6 may be important in VSMC biology and may involve signaling by its receptor, Axl, in conjunction with G protein receptor-mediated signaling pathways.

In addition to its biological implications in cultured VSMC, the function of GAS6 has been investigated in several other cell systems. In 3T3 cells, it was shown that GAS6 mediates both mitogenic and survival activities through ARK, the murine homologue of Axl (20, 21). This mitogenic effect is also observed in cultured human Schwann cells where GAS6 is found to stimulate DNA synthesis in these cells (22). By contrast, in murine hematopoietic 32D cells, we have demonstrated that exogenous GAS6 stimulation results in Axl autophosphorylation but no mitogenic or anti-apoptotic responses (23). In these cells, GAS6 promotes Axl-mediated adhesion, suggesting adhesion as one function for the GAS6-Axl interaction (24). Thus, the GAS6-associated biology appears complex and may be dependent upon the cellular context in which Axl and GAS6 operate.

In light of the findings that GAS6 appears important in the growth regulation of VSMC, we sought to investigate the effect of Axl-GAS6 interactions on other biological consequences in these cells. Here, we describe that in both rat and human primary VSMC, GAS6 can act as a chemoattractant for migration in modified Boyden chambers (25, 26). The chemotactic

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[1] The abbreviations used are: VSMC, vascular smooth muscle cells; ECD, extracellular domain; AoSMC, aortic smooth muscle cells; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; PDGF, platelet-derived growth factor.

[2] M. G. Melaragno, D. A. Wuthrich, V. Poppa, B. C. Berk, and M. A. Corson, submitted for publication.

nature of the GAS6-induced migration is evidenced in that a GAS6 gradient is necessary for this action. The specificity of GAS6-induced migration was further confirmed in that addition of purified Axl extracellular domain (ECD) attenuates migration. In agreement with previous studies in rat VSMC, GAS6 alone does not induce proliferation of human AoSMC (aortic smooth muscle cells). However, in response to GAS6, human AoSMC overexpressing Axl augment cell migration whereas ectopic expression of the Axl kinase dead mutant reduces the ligand-associated migration. Thus, these results strongly suggest that GAS6-Axl interactions induce chemotaxis of VSMC.

EXPERIMENTAL PROCEDURES

VSMC Cultures—Primary rat aortic VSMC were prepared and established essentially as described by Haller *et al.* (27). Briefly, thoracic aortas from young Sprague-Dawley rats were stripped of fatty and connective tissues and endothelium. The aortas were minced under aseptic conditions and treated with a combination of collagenase, elastase, and trypsin inhibitors as described (27). Primary rat aortic SMC were maintained in Dulbecco's modified Eagle's medium (DMEM-H) supplemented with 10% fetal bovine serum and penicillin/streptomycin (Life Technologies, Inc.). The phenotype of the primary VSMC was confirmed by staining the cells with smooth muscle-specific α-actin antibodies (Sigma) (27). Primary AoSMC were purchased from Clonetics, Inc. and maintained according to the provided instructions. Both rat and human cells for the experiments described in this study were used at passages 2–8.

Migration Assays—Migration assays were performed with modified Boyden chambers as described (26). Twenty-four well polyvinylidene difluoride filter inserts (8-μm pore diameter, Beckon Dickinson) were coated with a phosphate-buffered saline solution containing 100 μg/ml type I collagen (UBI) (26). To assemble modified Boyden chambers, the coated inserts were dropped into individual wells containing various factors in 0.8 ml of DMEM-H. Primary VSMC were gently trypsinized, counted, and allowed to recover in DMEM-H complete media for 1 h at room temperature with gentle rocking. Cells were then gently spun down and resuspended in DMEM-H containing 0.1% BSA at 1×10^5 cells/ml. Aliquots of 0.5 ml of cells were then transferred into the upper well of each assembled chamber. The chambers were incubated for 16 h at 37 °C in an atmosphere of 5% CO_2. Migration assays were also performed using 48-well micro-chemotaxis chambers (NeuroProbe, Inc., Cabin John, MD) as described (25). Similar results were obtained following shortened incubation periods (4 h) confirming the reproducibility of the assay.

Measurement of DNA Synthesis—Human AoSMC were seeded in 24-well plates and cultured in media recommended by the manufacturer (Clonetics, Inc.). At confluence, cells were rendered quiescent by serum starvation for 48–72 h. Following replacement of the culture medium with fresh medium, cells were stimulated with GAS6, PDGF, or BSA for re-entry of cell cycle. At various time points following ligand stimulation, cells were pulsed with 1.5 μCi/well [^{3}H]thymidine for 4 h. [^{3}H]Thymidine incorporation was measured after trichloroacetic acid precipitation as described (2).

Generation of Axl-overexpressing AoSMC Cell Lines—For retroviral infection of primary human AoSMC, amphotropic viruses were harvested from conditioned media of PA317 packaging cells following transfection of various Axl constructs (24).[3] Of three Axl constructs generated in the retroviral-based vector, pLXSN, the wild-type and the kinase dead (substitution of Arg for Lys at amino acid position 567) Axl constructions have been described (24).[3] The 1B1 construct contains a wild-type Axl cDNA sequence from which further Axl overexpression is induced by Moloney murine leukemia virus promoter mutations (data not shown). G418-resistant clones were selected and pooled to established cell lines. Passaging of these cells was kept to a minimum prior to protein analysis and migration assays to ensure the integrity of primary cell properties.

Immunoprecipitation and Western Blot Analysis—For Axl autophosphorylation assays, human AoSMC were grown to confluence in 150-mm plates and subjected to serum starvation for 48–72 h in DMEM-H. GAS6 stimulation was performed as described (23). Following 10 min stimulation at 37 °C, cells were washed with ice-cold phosphate-buffered saline and lysed in ice-cold HNTG lysis buffer (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 1.5 mM $MgCl_2$, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 mM Na_3VO_4, 0.15 TIU/ml aprotonin). Preparation of cell lysates for immunoprecipitation with anti-Axl polyclonal antibodies (1:250) as well as Western blot analysis were performed as described (23). Following electrophoretic transfer onto polyvinylidine difluoride membrane (Millipore), the phosphorylation status of the Axl receptor was examined by incubating the membrane with anti-phosphotyrosine antibodies (PT-66, 1:1000) (Sigma) followed by horseradish peroxidase-conjugated, donkey anti-mouse secondary antibodies (1:5000) (Amersham) (23). Western blots were developed with ECL reagents (Amersham). For analysis of ectopic Axl expression, AoSMC were lysed as described above. Equal amounts of total proteins (50–100 μg) from each cell line were then analyzed for Axl expression by Western blotting using anti-Axl polyclonal antibodies (1:1000) (23).

Production and Purification of Axl-ECD from Sf9 Insect Cells—The extracellular domain of the Axl receptor (Axl-ECD) was expressed in Sf9 insect cells using the baculovirus expression system (6, 24). Production and purification of the Axl-ECD protein was carried out as described (6, 24).

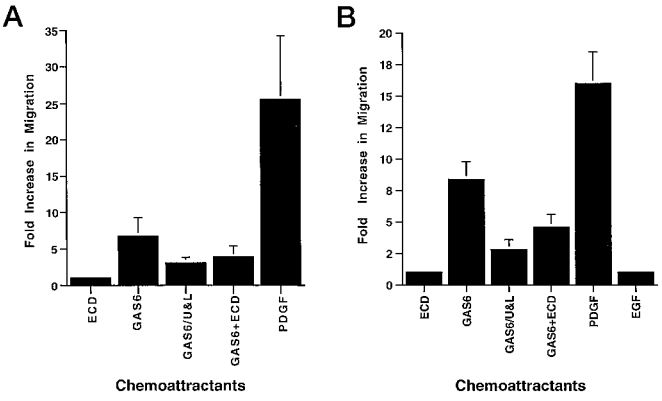

FIG. 1. **GAS6 induces directed migration of rat (*A*) and human (*B*) VSMC.** Migration assays were performed as described under "Experimental Procedures." Modified Boyden chambers were assembled by adding DMEM-H containing BSA (200 ng/ml), ECD: purified Axl-ECD (200 ng/ml), human recombinant GAS6 (200 ng/ml), a combination of Axl-ECD and GAS6, epidermal growth factor (*EGF*) (100 ng/ml) (UBI), or PDGF-BB (30 ng/ml) (Genzyme) in a total volume of 0.8 ml to the lower well of the chambers. Filters were coated with collagen I (100 μg/ml) and then placed in the apparatus. Fifty thousand cells resuspended in 0.5 ml of DMEM-H were then added to the upper well of the chamber. For experiments assaying the requirement for a GAS6 gradient, cells containing 200 ng/ml GAS6 in DMEM-H were placed in the upper well of the chamber (*GAS6/U&L*). Following 16 h of incubation at 37 °C, cells were fixed, stained, and counted at × 100–200 magnification. Each *bar* represents the average (±S.D.) fold increase in migration (subtracted from BSA control) as determined from three to five independent experiments. Migration assays were also performed using 48-well micro-chemotaxis chambers as described (NeuroProbe, Inc.) (25). Reproducible results were obtained following shortened incubation periods (4 h) (see "Experimental Procedures").

RESULTS AND DISCUSSION

Directed migration of VSMC from tunica media to intima and subsequent proliferation of neointima are two key events involved in atherogenesis following both the mechanical injury and inflammatory responses of the arteries (28, 29). Given the findings that GAS6 may play a role as a cofactor in proliferation for rat VSMC, we wished to investigate whether GAS6 also possessed chemotactic properties in these cells. To examine whether GAS6 could induce chemotaxis of VSMC, migration assays in modified Boyden chambers were conducted (Refs. 25 and 26, also see "Experimental Procedures"). As shown in Fig. 1*A*, human recombinant GAS6 induced chemotaxis of rat VSMC at approximately 6-fold greater than controls. A dose-response experiment showed that 200 ng/ml GAS6 induced the maximal level of migration, a concentration of GAS6 comparable to that observed for inducing maximal Axl autophosphorylation (data not shown) (6). In comparison, PDGF-BB, the most

[3] A. Burchert, E. C. Attar, P. McCloskey, Y.-W. C. Fridell, and E. T. Liu, in press.

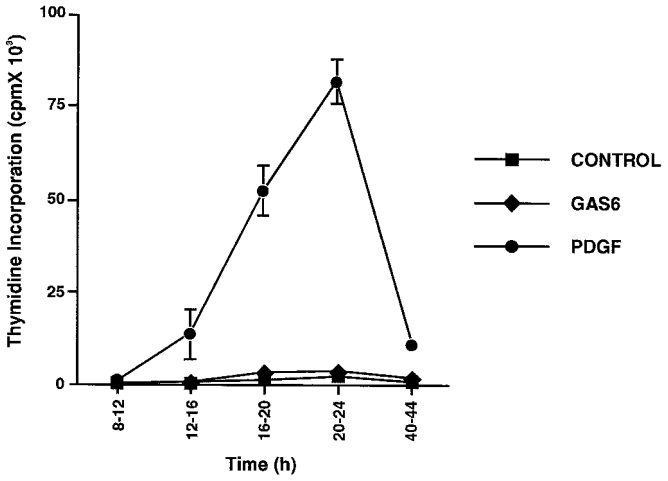

FIG. 2. **GAS6 does not induce mitogenesis in human AoSMC.** In 24-well plates, confluent human VSMC were rendered quiescent after incubation in DMEM-H for 48 h. For ligand stimulation, fresh DMEM-H containing BSA (200 ng/ml), GAS6 (200 ng/ml), or PDGF (50 ng/ml) was added to each well. At the indicated times, cells were pulsed with 1.5 μCi/well [^{3}H]thymidine for 4 h.

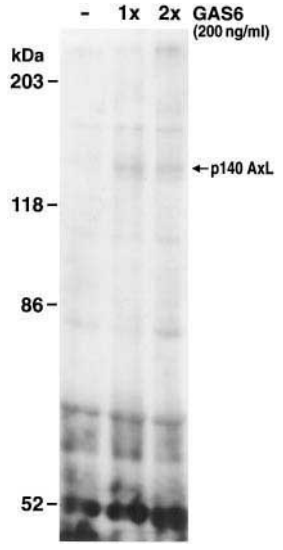

FIG. 3. **Axl phosphorylation in response to exogenous GAS6 stimulation in human AoSMC.** Primary human AoSMC were mock stimulated (−), stimulated with 1 × GAS6 (200 ng/ml) or 2 × GAS6 (400 ng/ml) for 10 min at 37 °C. Cells were lysed and subjected to immunoprecipitation with an anti-Axl polyclonal antibody at 1:250. Subsequent Western blotting analysis with an anti-Tyr(P) antibody at 1:1000 (PT-66, Sigma) was performed as described under "Experimental Procedures." Molecular weight markers are indicated. The position of the p140 Axl is indicated.

potent chemoattractant for VSMC described to date, induced chemotaxis of these cells 4–5-fold greater than that induced by GAS6 (Fig. 1*A*) (19, 28). The chemotactic but not chemokinetic effect of GAS6 on these cells was evidenced by the fact that a GAS6 gradient induced directed migration. When the same concentration of GAS6 was included in both upper and lower wells of the chambers, migration of these cells was reduced to one-third to one-half of that seen when gradients were maintained (Fig. 1*A*). Furthermore, this effect can be attenuated by the addition of soluble, recombinant Axl extracellular domain protein (Axl-ECD) at a 1:1 molar ratio in the lower well of the chambers indicating that GAS6 serves as the signal for chemotaxis of these cells (6, 24).

To verify that the chemotactic effect induced by human GAS6 in rat VSMC is physiologically relevant, we investigated whether human recombinant GAS6 would induce directed migration of primary human AoSMC in a similar fashion. As expected, human AoSMC migrated in response to GAS6 in the same manner as observed in rat VSMC (Fig. 1*B*). Chemotaxis of these cells toward GAS6 was dependent upon the presence of a GAS6 concentration gradient between the upper and lower wells of the modified Boyen chamber (Fig. 1*B*). Again, GAS6-induced chemotaxis was reduced by the presence of purified Axl-ECD. Finally, epidermal growth factor, a known mitogen for VSMC that has no chemoattractant properties showed no effect in inducing migration of these cells demonstrating that mitogenicity and cell migration can be separated by these *in vitro* assays (Fig. 1*B*). Thus, GAS6 functions as a chemoattractant for VSMC migration *in vitro*.

To determine if GAS6 alone can act as a mitogen in human AoSMC, DNA synthesis was measured in these cells following GAS6 stimulation. As shown in Fig. 2, marginal mitogenic effects were detected in quiescent cells when stimulated with GAS6 (1.5-fold increase as compared with control) whereas PDGF-BB treatment induced potent mitogenesis of these cells (Fig. 2). Although previous studies indicated that GAS6 potentiates a 2-fold increase in proliferation of rat VSMC in the presence of thrombin, we did not observe such an increase in cultured human AoSMC using human GAS6 under the same conditions (data not shown) (2). Therefore, we hypothesize that the primary function of human GAS6 *in vivo* is to induce migration of AoSMC without stimulating proliferation of these cells.

We sought to identify specific ligand-receptor interactions responsible for GAS6-induced VSMC migration. Following GAS6 stimulation, Axl-specific antibodies were used to immunoprecipitate lysates from quiescent AoSMC. As shown in Fig. 3, when probed with anti-phosphotyrosine antibodies, GAS6 stimulation resulted in Axl autophosphorylation. Thus, GAS6-Axl interactions may be associated with GAS6-induced migration of human AoSMC. Several attempts were made to determine the phosphorylation status of Mer and Sky following GAS6 stimulation in AoSMC. However, due to the lack of suitable Mer and Sky antibodies for these analyses, the exact involvement of these two receptor kinases in this process remains to be determined. To further test the hypothesis that Axl mediates GAS6-induced AoSMC migration, primary human AoSMC ectopically expressing Axl were generated.[3] Migration assays were then performed to assess the ability of these Axl-expressing cells to migrate in the presence of GAS6 in the lower well of the modified Boyden chamber (25, 26). Two AoSM cell lines overexpressing the wild-type Axl were generated. As demonstrated in Fig. 4*A*, Axl/AoSMC expressed moderately higher Axl than the parental cells whereas a dramatic overexpression of Axl was achieved in 1B1/AoSMC.[3] When tested for migration, moderate Axl-overexpressors (Axl/AoSMC) exhibited a 2.5–3-fold increase in GAS6-dependent migration as compared with parental AoSMC (Fig. 4*B*). By contrast, 1B1/AoSMC demonstrated a further increase (up to 5-fold) in numbers of migrated cells as compared with parental AoSMC in response to GAS6 (Fig. 4*B*). In addition, the increased migration seen in Axl-overexpressing AoSMC was again attenuated by the application of the Axl-ECD in the lower well of the chambers (data not shown). To further confirm the direct involvement of Axl in this process, AoSMC expressing the kinase dead mutant of Axl (AxlK567R) were generated (Fig. 4*A*) (24). Significantly, when tested for chemotaxis, these cells exhibited reduced migration in response to GAS6 as compared with the uninfected parental AoSMC (Fig. 4*B*). In the same assays, the parental AoSMC and the ectopic Axl-expressing AoSM cells had identical migratory responses to PDGF-BB, indicating the specificity of Axl-de-

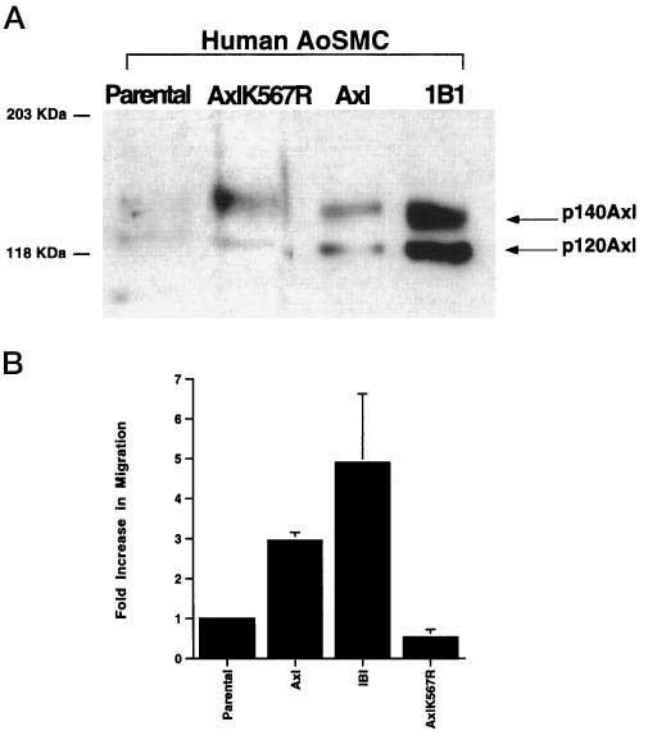

FIG. 4. **Ectopic expression of wild-type and the kinase dead mutant of Axl in human AoSMC (*A*) and migration assays (*B*) of these cells.** *A*, 100 μg of total protein from each cell line was separated on a 7.5% SDS-polyacrylamide gel. Western blot analysis with anti-Axl antibody (1:1000) was performed. AxlK567R is the kinase dead Axl mutant. *Axl* contains the wild-type Axl cDNA sequence. *1B1* construct containing a wild-type Axl cDNA sequence overexpresses the Axl protein. *B*, for migration assays, modified Boyden chambers were assembled by adding either BSA (200 ng/ml) or human recombinant GAS6 (200 ng/ml) in DMEM-H to the lower well of the chambers. Filters were coated with collagen I and then placed in the apparatus. Fifty thousand cells resuspended in DMEM-H were then added to the upper well of the chamber. Following 16 h of incubation at 37 °C, cells were fixed, stained, and counted at × 100–200 magnification. Each *bar* represents the average (±S.D.) fold increase in migration (subtracted from migration of parental AoSMC) as determined from three independent experiments.

pendent effects on migration in these cell lines (data not shown). Taken together, these results strongly indicate that the Axl receptor mediates GAS6-induced migration of AoSMC *in vitro* and that the kinase activity of the Axl receptor is involved in migration in response to GAS6. Furthermore, the fact that AxlK567R bearing AoSM cells showed reduced levels of GAS6-induced migration suggests that other Axl-related tyrosine kinase receptors may not be playing a significant role in this process.

Melaragno and co-workers recently showed an up-regulation of Axl expression 7–14 days following balloon injury of rat carotid arteries.[2] This increased Axl expression correlates well with the time course of neointima thickening due to medial VSMC migration post-injury (29). Furthermore, the immunohistochemical staining of injured vessels showed increased Axl expression localized to the neointima.[2] Northern blot analysis on denuded vessels following balloon injury of rat carotid arteries has revealed an increase of GAS6 transcripts in these tissues.[4] In light of these findings, we suggest the following hypothesis for Axl and GAS6 involvement in response to arterial injury: following arterial injury, increased local GAS6 concentration permits formation of GAS6 gradient. This gradient may then induce the migration of medial smooth muscle cells to the intima. The subsequent up-regulation of Axl expression post-injury, perhaps influenced by G protein-coupled agonists on site, thus renders VSMC more sensitive to GAS6 for proliferation. These processes may, in turn, contribute to atherosclerotic plaque formation and arterial restenosis.

In summary, we have uncovered a novel function for human recombinant GAS6 as a chemoattractant in cultured rat and human VSMC. Significantly, we have demonstrated that GAS6-induced migration appears to be mediated by its receptor, Axl. Further insights into atherogenesis can be gained by determining the signaling pathways activated by GAS6-Axl interactions in these cells. Thus, interventions may be devised to block specific downstream effectors following Axl activation to alleviate the consequences of vascular damage.

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[4] V. Lindner, personal communication.

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